Cognitive Strategy-Specific Increases in Phosphorylated cAMP Response Element-Binding Protein and c-Fos in the Hippocampus and Dorsal Striatum

Paul J. Colombo,[1,2] **Jennifer J. Brightwell,**[1] **and Renee A. Countryman**[2]
[1]Neuroscience Program and [2]Department of Psychology, Tulane University, New Orleans, Louisiana 70118

Extensive research has shown that the hippocampus and striatum have dissociable roles in memory and are necessary for "place" and "response" learning, respectively. In the present study, rats were trained on a cross maze task that could be solved by either a place or a response strategy, and the strategy used was determined by a probe trial. Phosphorylated cAMP response element-binding protein (pCREB) and c-Fos immunoreactivity (IR) were measured in the hippocampus and striatum either immediately or 1 hr after cross maze training. Immediately after training, pCREB-IR and c-Fos-IR were significantly higher in the hippocampus and striatum of trained rats than in control rats matched for motor activity, but the increase was independent of the strategy revealed at probe. One hour after training, however, pCREB-IR and c-Fos-IR were sustained in the hippocampal pyramidal and granule cell layers of place learners but returned to basal levels among response learners. In addition, pCREB-IR was sustained in the dorsomedial and dorsolateral striatum of response learners but returned to basal levels among place learners. There were no differences between place and response learners in c-Fos-IR in the striatum at either time point. The present results indicate that cross maze training causes an initial activation of transcription factors in both the hippocampus and striatum. Formation of memory for a place strategy, however, is related to sustained phosphorylation of CREB and expression of c-Fos for at least 1 hr in the hippocampus, whereas formation of memory for a response strategy is related to phosphorylation of CREB in the striatum.

Key words: **cAMP response element-binding protein; CREB; c-Fos; place learning; response learning; hippocampus; dorsal neostriatum; cross maze**

Introduction

Distinct memory functions are attributed to the hippocampal formation and the neostriatum in humans (Knowlton et al., 1996; Maguire et al., 1998; Casey et al., 2002), nonhuman primates (Teng et al., 2000; Fernandez-Ruiz et al., 2001), and rats (Kesner and Beers, 1988; Kesner et al., 1993; McDonald and White, 1993; Packard et al., 1994; Compton, 2001). Lesions or pharmacological manipulations that alter the function of the hippocampal formation or the neostriatum provide the primary evidence that these two brain systems are specialized for different types of memory. In rats, damage to the hippocampal formation selectively impairs formation of memory for the relationships among stimuli (Eichenbaum et al., 1990; Eichenbaum, 2001), whereas damage to the neostriatum tends to impair acquisition of associations between stimuli and motor responses (Colombo et al., 1989; Packard and White, 1990; Packard and McGaugh, 1992; McDonald and White, 1994; Golf Racht-Delatour and El Massioui, 1999). Specialized mnemonic functions attributed to the hippocampus and striatum, respectively, include memory for spatial and nonspatial information (Packard and McGaugh, 1992, 1996), memory for allocentric (place) and egocentric (response) information (Kesner and Beers, 1988; Packard, 1999), and declarative and procedural memory (DeCoteau and Kesner, 2000). Although there is compelling evidence that the contributions of the hippocampus and striatum to memory formation can be dissociated, the relationship between the systems during normal operations is not understood. One recent report suggests that the two systems may operate in temporal sequence (Packard, 1999).

Members of the cAMP response element-binding protein (CREB) family of transcription factors have been implicated in the formation of long-term memory (Lamprecht and Dudai, 1996; Lamprecht et al., 1997). Suppression of CREB protein by administration of antisense oligodeoxynucleotides (Guzowski and McGaugh, 1997) or genetic knock-out (Bourtchuladze et al., 1994) impairs spatial memory, whereas overexpression of CREB can enhance the formation of long-term memory (Josselyn et al., 2001). CREB phosphorylation is increased by exposure to a novel environment (Vianna et al., 2000), contextual fear conditioning (Stanciu et al., 2001), inhibitory avoidance (Bernabeu et al., 1997; Cammarota et al., 2000; Taubenfeld et al., 2001), and radial arm maze training (Mizuno et al., 2002). Stimuli that increase cAMP or Ca^{2+}-dependent protein kinase activity phosphorylate CREB (Gonzalez and Montminy, 1989; Dash et al., 1991), and phosphorylated CREB (pCREB) stimulates the expression of immediate-early genes, including the transcription factor c-Fos (Sheng and Greenberg, 1990). Learning-induced expression of c-Fos is implicated in visual recognition memory (Wan et al., 1999), fear conditioning (Milanovic et al., 1998; Radulovic et al., 1998), spatial working (Vann et al., 2000) and reference memory

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Correspondence should be addressed to Paul J. Colombo, Tulane University, Department of Psychology, New Orleans, LA 70118. E-mail: pcolomb@tulane.edu.
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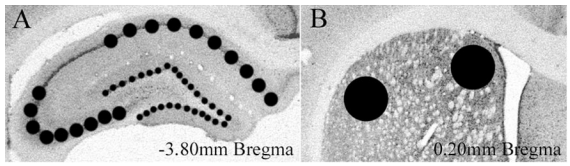

Figure 1. *A*, Immunoreactive cells were counted within sampled areas of the dorsal hippocampus. These included the pyramidal cell layers CA1 and CA3 (medium circles) and the granule cell layers of the dentate gyrus (small circles). *B*, Sampled areas of the dorsomedial and dorsolateral striatum (large circles).

(Qiang et al., 1999), brightness discrimination (Tischmeyer et al., 1990), and avoidance learning (Qiang et al., 1999; Cammarota et al., 2000).

In the present study, rats were trained on a cross maze task that could be solved by either a hippocampus-dependent place strategy or a dorsal striatum-dependent response strategy, and the strategy used was determined during a probe trial. Levels of pCREB and c-Fos immunoreactivity (IR) were measured in the hippocampus and striatum either immediately or 1 hr after cross maze training.

Materials and Methods

Eighty-two naive male Long–Evans hooded rats (weight, 250–275 gm) were housed individually in a temperature-controlled environment with a 12 hr light/dark cycle (lights on at 7:00 A.M.) and *ad libitum* access to food and water. All behavioral testing was conducted during the light phase of the cycle.

The behavioral apparatus was an eight arm radial maze (Lafayette Instruments, Lafayette, IN) with black metal floors and clear Plexiglas walls. The arms of the cross maze (10 cm wide × 70 cm long × 20 cm high) had recessed food wells at the end and were separated from an octagonal center compartment (33 cm). The maze was located in a testing room that contained several extramaze cues and was sanitized between rats and before all probe trials to inhibit intramaze olfactory cues.

Animals were handled extensively (5 min each per day) for 2 weeks from the time of arrival to the first day of habituation. One week before maze testing, animals were reduced to 85% of their free-feeding weights over 7 d and maintained at this weight throughout the experiment. One day before habituation, animals received 4 gm of Froot Loops cereal (Kellogg, Battle Creek, MI) in addition to sufficient chow to maintain the 85% target weight and reduce neophobic reactions to the Froot Loops during training trials.

Animals were habituated for 3 d with one 5 min trial per day. On day 1, the rats were placed into the south arm of the maze with two additional arms open at right angles (west and east) and with Froot Loops broken into thirds and scattered throughout all three arms. On day 2, the Froot Loops were placed only in the east and west arms of the maze, and on day 3, the Froot Loops were placed only in the recessed food wells at the ends of the east and west arms. Training and testing were completed on the fourth day. Rats were released from the end of the south arm and allowed to enter one arm (neither of which was baited), and the arm opposite the one chosen was baited for all subsequent trials. On training trials, rats were allowed 2 min to obtain the food reward of one-third of a Froot Loop. Entries into the unbaited arm of the maze were scored as incorrect responses, and entries into the baited arm of the maze were scored as correct responses. An entry was defined as all four feet crossing into an arm, and rats were allowed to enter one arm only per trial, after which they were placed back into their holding cage for a 30 sec intertrial interval. All rats were trained to a criterion of 9 of 10 correct choices (range, 11–44 trials), after which they were released from the arm opposite the original start location (north) for a single probe trial. Rats that reentered the arm rewarded during training were categorized as place learners, whereas rats that made the same turning response as that re-

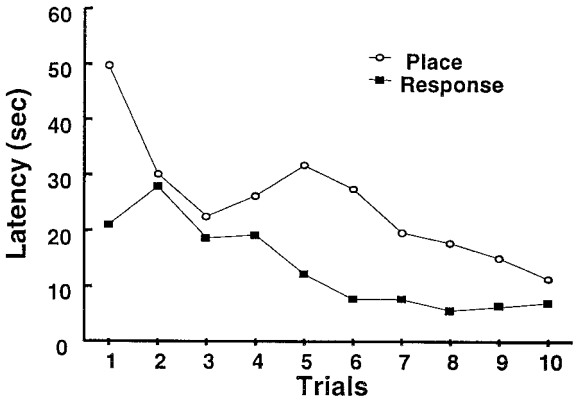

Figure 2. Latency to complete the last 10 criterion trials for place learners ($n = 27$) and response learners ($n = 23$).

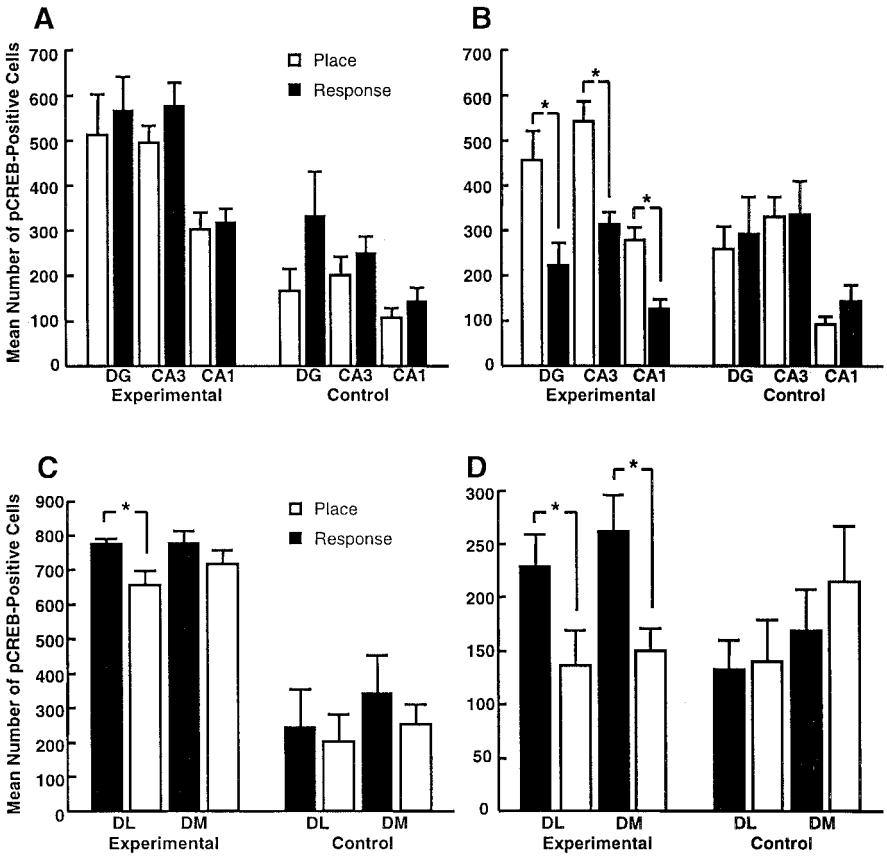

Figure 3. Numbers of pCREB-positive cells in the hippocampus (*A, B*) and striatum (*C, D*) of place learners, response learners, and controls matched to the number and duration of trials of place and response learners either immediately (*A, C*) or 1 hr (*B, D*) after completion of training. DG, Dentate gyrus; DL, dorsolateral striatum; DM, dorsomedial striatum. *$p < 0.05$.

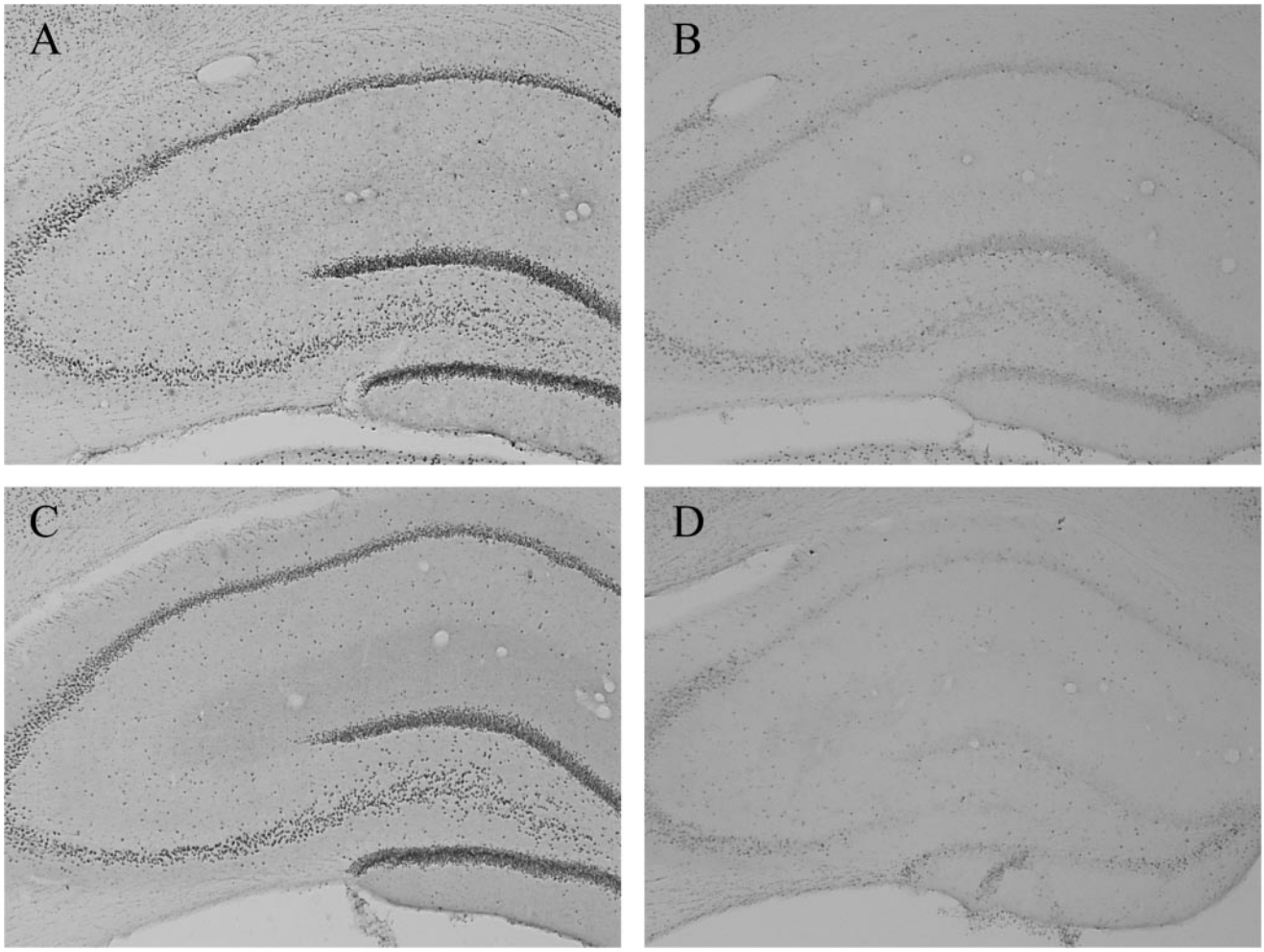

Figure 4. *A*, pCREB-IR in the hippocampus of a representative place learner immediately after training. *B*, pCREB-IR in the hippocampus of a representative motor control immediately after training. pCREB-IR in the hippocampus of a representative response learner immediately (*C*) and 1 hr (*D*) after training.

warded during training and thus entered the arm opposite the one rewarded during training were categorized as response learners. Previous research (Restle, 1957; Packard, 1999) and pilot studies conducted in this laboratory (data not shown) indicate that factors such as the amount of training, salience of extramaze cues, and amount of illumination influence the numbers of rats characterized as place and response learners based on performance during the probe trial. In the present study, probe trials revealed that approximately one-half of the trained rats used a place strategy ($n = 27$) and one-half used a response strategy ($n = 23$). Rats in control conditions were matched to the numbers and durations of trials of both place ($n = 16$) and response ($n = 16$) learners. This group was included to test whether differences in pCREB or c-Fos between place and response learners were attributable to differences in cognitive factors or to differences in locomotor or other performance factors. Control rats were released from the south arm on all trials and could access the north arm only, whereas experimental rats were released from the south arm on all trials except the probe trial. Thus, control rats were matched to experimental rats in all aspects of the task except that they had only one arm available for food reward, whereas experimental rats had to choose between two.

Either immediately or 1 hr after the probe trial, animals were injected with a ketamine–xylazine solution (1.65 mg/kg) and perfused transcardially with ice-cold 0.1 M PBS, pH 7.4, and $NaNO_3$ followed by ice-cold 4% paraformaldehyde in 0.1 M PBS. Brains were removed and postfixed in 4% paraformaldehyde for 3 hr and then transferred to a 20% sucrose and 0.1 M phosphate buffer cryoprotectant overnight at 4°C. Forty micrometer coronal sections were taken beginning at the posterior end of the anterior olfactory nucleus and extending throughout the hippocampus. Sections were collected in cryopreservative and frozen at −70°C. For each subject, four sections were selected at approximately bregma 0.2 mm for striatum and four sections at bregma −3.8 mm for the hippocampus (Fig. 1) and immunostained as described below. Net wells (24 mm; Corning, Corning, NY) were used to wash tissue sections several times in 0.05 M PBS and then once in 1% normal goat serum (NGS), 0.02% Triton X-100 (TX), and 1% H_2O_2 in PBS for 10 min to inhibit endogenous peroxidase. Sections were blocked for 15 min in a 2% NGS and 0.4% TX solution in PBS followed by incubation in 1% NGS and 0.4% TX in PBS containing either c-Fos rabbit polyclonal antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) or pCREB rabbit polyclonal antibody (1:1000; Upstate Biotechnology, Lake Placid, NY) for 48 hr at 4°C. Sections were washed four times with 0.05 M PBS for 15 min each before a 1 hr incubation in biotinylated goat anti-rabbit secondary antibody (1:400 in 1% NGS and 0.2% TX PBS; Santa Cruz Biotechnology). Sections were washed in 0.05 M PBS three times for 5 min each and then processed with avidin-biotinylated horseradish peroxidase complex in PBS (Elite Kit; Vector Laboratories, Burlingame, CA) for 45 min at room temperature. Sections were washed four times for 15 min each in PBS, and the reaction was visualized with diaminobenzidine (DAB substrate kit; Vector Laboratories). The reaction was stopped by washing three times for 10 min each in cold 0.01 M PBS. Sections were mounted on slides, allowed to dry overnight, and plated under coverslips. Nuclear immunoreactivity was quantified by the two focal plane method of Brown et al. (1998). Sampling templates of consistent area were established, and immunoreactive cells were counted in CA1, CA3,

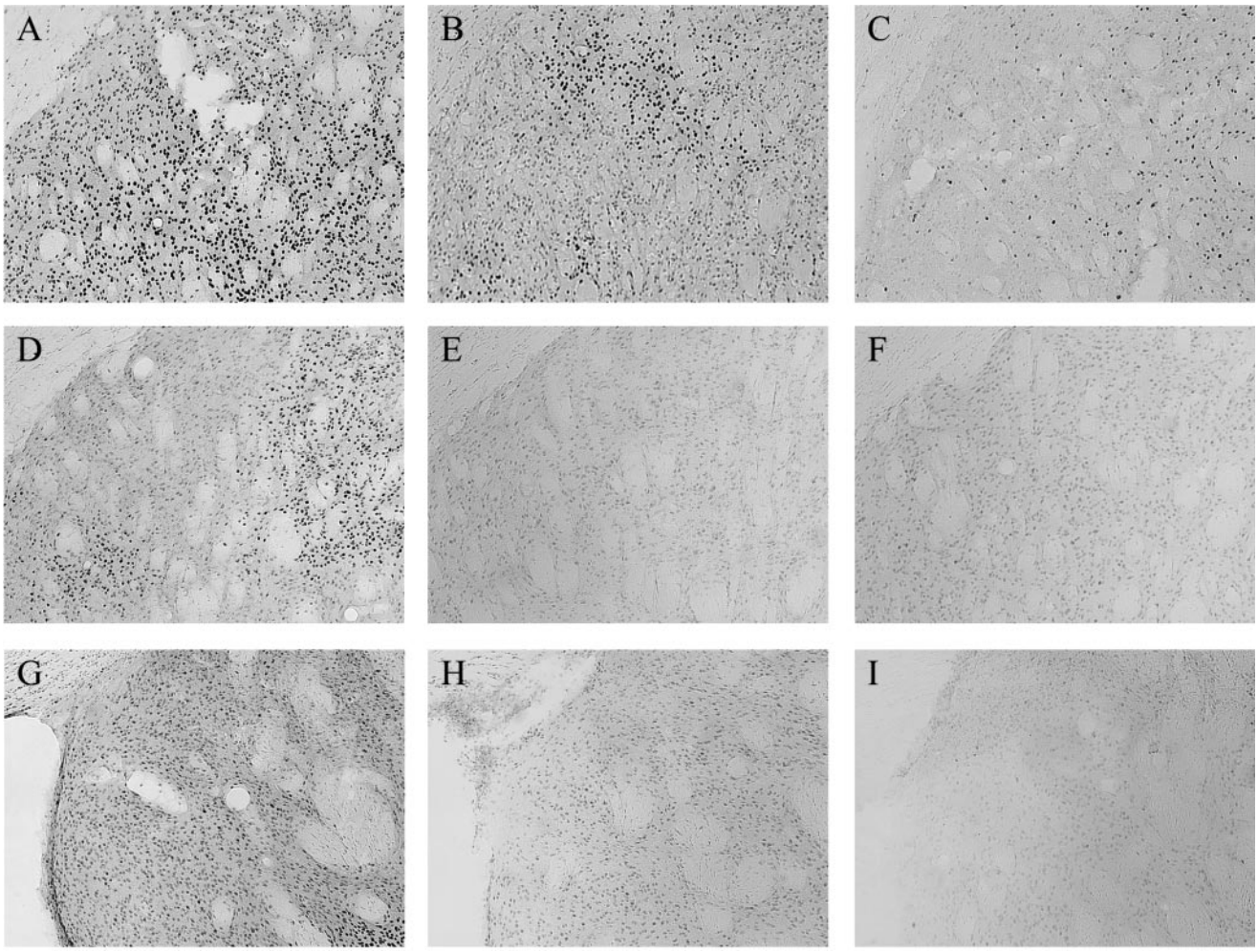

Figure 5. Representative pCREB-IR immediately after training in the dorsolateral striatum of a response learner (*A*), place learner (*B*), and motor control (*C*). Representative pCREB-IR 1 hr after training in the dorsolateral striatum of a response learner (*D*), place learner (*E*), and motor control (*F*). Representative pCREB-IR 1 hr after training in the dorsomedial striatum of a response learner (*G*), place learner (*H*), and motor control (*I*).

and the dentate gyrus of the dorsal hippocampus, as well as the dorsomedial and dorsolateral striatum (Fig. 1).

Results

ANOVA revealed no difference in the number of trials to criterion between place and response learners ($F_{(1,48)} = 0.01$; $p = 0.92$). The mean number of trials to criterion was 18.9 ± 1.1 SEM for place learners and 18.7 ± 1.7 SEM for response learners. In contrast, the total time in the maze and the average trial duration over the last 10 criterion trials were greater among place learners than among response learners ($F_{(1,48)} = 6.86$, $p = 0.01$ and $F_{(1,48)} = 7.83$, $p = 0.007$, respectively) (Fig. 2).

Relationships between learning and levels of pCREB-IR and c-Fos-IR were analyzed independently for the hippocampus and striatum. Multivariate ANOVAs (MANOVAs) were conducted with levels of pCREB-IR and c-Fos-IR in CA1, CA3, and the dentate gyrus of the hippocampus and dorsomedial and dorsolateral striatum as dependent variables. Independent variables were training condition (trained or control), type of strategy revealed at probe test (place or response), and interval between test and killing of the animal (immediate or 1 hr).

Overall MANOVA of pCREB in the hippocampus revealed significant main effects for training condition ($F_{(3,71)} = 20.4$; $p < 0.001$), test-to-killing interval ($F_{(3,71)} = 2.96$; $p = 0.04$), and a training condition by test-to-killing interval interaction ($F_{(3,71)} = 3.92$; $p = 0.01$) (Figs. 3*A*,*B*, 4). Overall ANOVA of pCREB in the striatum revealed significant main effects for training condition ($F_{(2,71)} = 37.6$; $p < 0.001$), test-to-killing interval ($F_{(2,71)} = 60.2$; $p < 0.001$), and a training condition by test-to-killing interval interaction ($F_{(2,71)} = 28.7$; $p < 0.001$) (Figs. 3*C*,*D*, 5). Thus, trained rats had significantly more hippocampal and striatal pCREB-IR than motor controls immediately after training, and that effect was independent of whether rats showed place or response strategies. One hour after training, however, levels of pCREB remained significantly greater in the hippocampus of place learners than in that of motor controls ($F_{(1,20)} = 13.6$; $p = 0.001$), whereas there was no difference between levels of pCREB in the hippocampus of response learners compared with motor controls ($F_{(1,18)} = 0.35$; $p = 0.56$) (Fig. 3*B*). Of particular importance, place learners had higher levels of hippocampal pCREB than response learners 1 hr after training. These differences were found throughout the principal cell layers of the hippocampus and included the dentate gyrus ($F_{(1,24)} = 7.8$; $p = 0.01$), CA1 ($F_{(1,24)} = 17.9$; $p < 0.001$), and CA3 ($F_{(1,24)} = 18.4$; $p < 0.001$). The converse pattern was found in the striatum 1 hr after training. That is, levels of pCREB remained significantly greater in the striatum of response learners than in that of motor controls

($F_{(1,18)} = 4.8$; $p = 0.04$), but there was no difference between levels of pCREB in the striatum of place learners and that of motor controls ($F_{(1,20)} = 0.78$; $p = 0.39$) (Fig. 3*D*). Response learners had significantly greater levels of pCREB than place learners in the dorsolateral striatum immediately ($F_{(1,21)} = 6.1$; $p = 0.02$) and 1 hr after training ($F_{(1,24)} = 4.3$; $p = 0.049$) and in the dorsomedial striatum ($F_{(1,24)} = 8.48$; $p = 0.008$) 1 hr after training (Fig. 3*C,D*).

Overall MANOVA of c-Fos in the hippocampus revealed a significant main effect of training condition ($F_{(3,63)} = 73.8$; $p < 0.001$). Thus, there was significantly more hippocampal c-Fos-IR in trained rats than in control rats immediately and 1 hr after training (Figs. 6*A,B*, 7). In the striatum, overall ANOVA of c-Fos revealed a significant main effect of test-to-killing interval ($F_{(2,71)} = 24.5$; $p < 0.001$) and a training condition by test-to-killing interval interaction ($F_{(2,71)} = 6.67$; $p = 0.002$). Thus, there was greater c-Fos-IR immediately after training than 1 hr after training. The training condition by test-to-killing interaction was attributable to greater c-Fos-IR among trained rats than among motor controls immediately after training ($F_{(2,37)} = 5.88$; $p = 0.006$) but not 1 hr after training (Fig. 6*C,D*). Place learners and response learners had equivalent levels of c-Fos-IR in the hippocampus immediately after training. At 1 hr after training, however, place learners had significantly higher levels of c-Fos-IR in the dentate gyrus and CA1 than response learners ($F_{(1,24)} = 4.3$, $p = 0.04$ and $F_{(1,24)} = 4.9$, $p = 0.01$, respectively) (Fig. 6*B*). There were no differences in striatal c-Fos-IR between place and response learners either immediately or 1 hr after training (Figs. 6*C,D*, 8). Representative images of pCREB and c-Fos immunoreactivity in the hippocampus and striatum of place learners, response learners, and controls are shown in Figures 4, 5, 7, and 8.

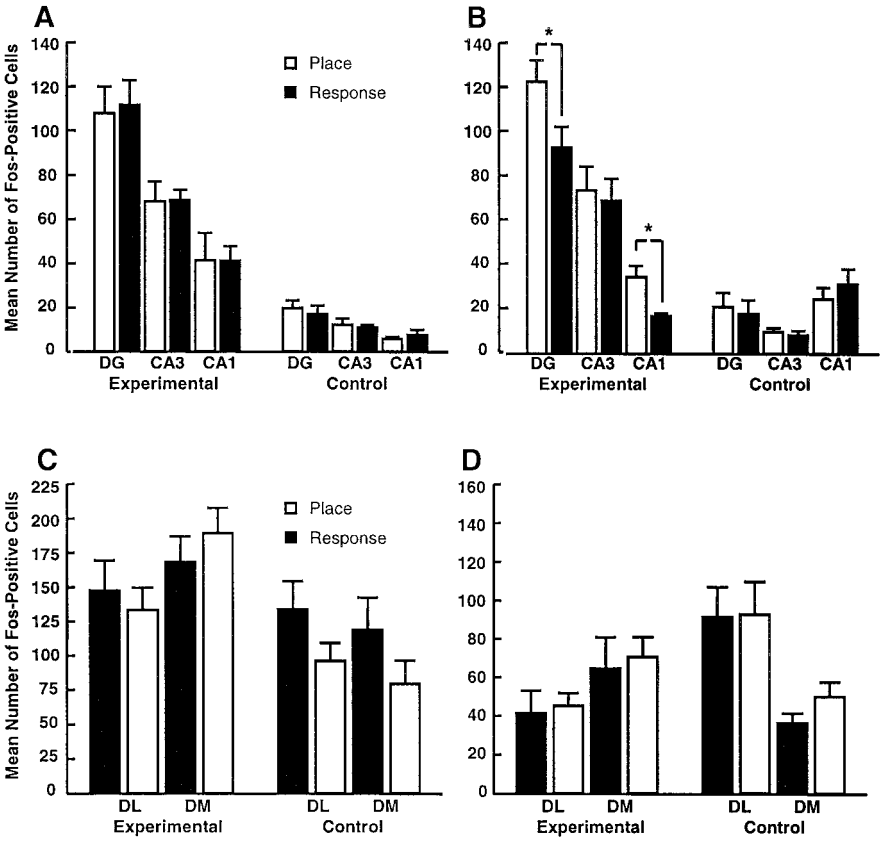

Figure 6. Numbers of c-Fos-positive cells in the hippocampus (*A, B*) and striatum (*C, D*) of place learners, response learners, and controls matched to the numbers and durations of trials of place and response learners either immediately (*A, C*) or 1 hr (*B, D*) after completion of training. DG, Dentate gyrus; DL, dorsolateral striatum; DM, dorsomedial striatum. *$p < 0.05$.

Discussion

The main findings of this study are twofold. First, rats trained to make a spatial discrimination in the cross maze have significantly more phosphorylation of CREB and expression of c-Fos in the hippocampus and striatum than control rats matched for motor activity and time in the maze. The activity of these transcription factors occurs initially in both the hippocampus and the striatum independently of whether rats use place or response strategies to solve the task. Second, regional activity of CREB, and to a lesser extent c-Fos, distinguishes place and response learners 1 hr after training. Specifically, pCREB-IR is greater in the dentate gyrus, CA1, and CA3 of place learners than in that of response learners. In contrast, pCREB-IR is greater in the dorsolateral and dorsomedial striatum of response learners than in that of place learners. Elevated c-Fos is observed in the dentate gyrus and CA1 of place learners compared with response learners 1 hr after training.

It is most likely that regional differences in pCREB and c-Fos between place and response learners are related to differences in cognitive factors rather than to differences in locomotor or other performance factors. This conclusion is based on our finding that levels of pCREB and c-Fos do not differ in either the hippocampus or the striatum of control rats matched to the numbers and durations of trials of place learners in comparisons with controls matched to response learners. Thus, differences in the numbers and durations of trials in the range observed between place and response learners are not sufficient to cause the differences in pCREB and c-Fos that we report here.

The current findings extend previous dissociations of the hippocampus and striatum resulting from localized lesions (Packard and McGaugh, 1992; Kesner et al., 1993; McDonald and White, 1993) or pharmacological manipulations (Packard and White, 1990; Packard and Teather, 1997, 1998; Packard, 1999). Specifically, functional measures of transcription factors involved in memory formation reveal localized activity in the hippocampus of place learners and in the striatum of response learners.

White and McDonald (2002) theorized that multiple parallel memory systems including the hippocampus and dorsal striatum access much of the same information, but that each system has a unique "processing style." The present results are consistent with this theory in that activation of the transcription factors CREB and c-Fos was observed initially in both the hippocampus and striatum immediately after training. One hour after training, however, sustained activation of the transcription factors was observed only in the brain region implicated in a particular type of memory storage. Sustained pCREB and c-Fos were observed in the hippocampus of place learners but not response learners, whereas sustained pCREB occurs in the dorsal striatum of response learners but not place learners.

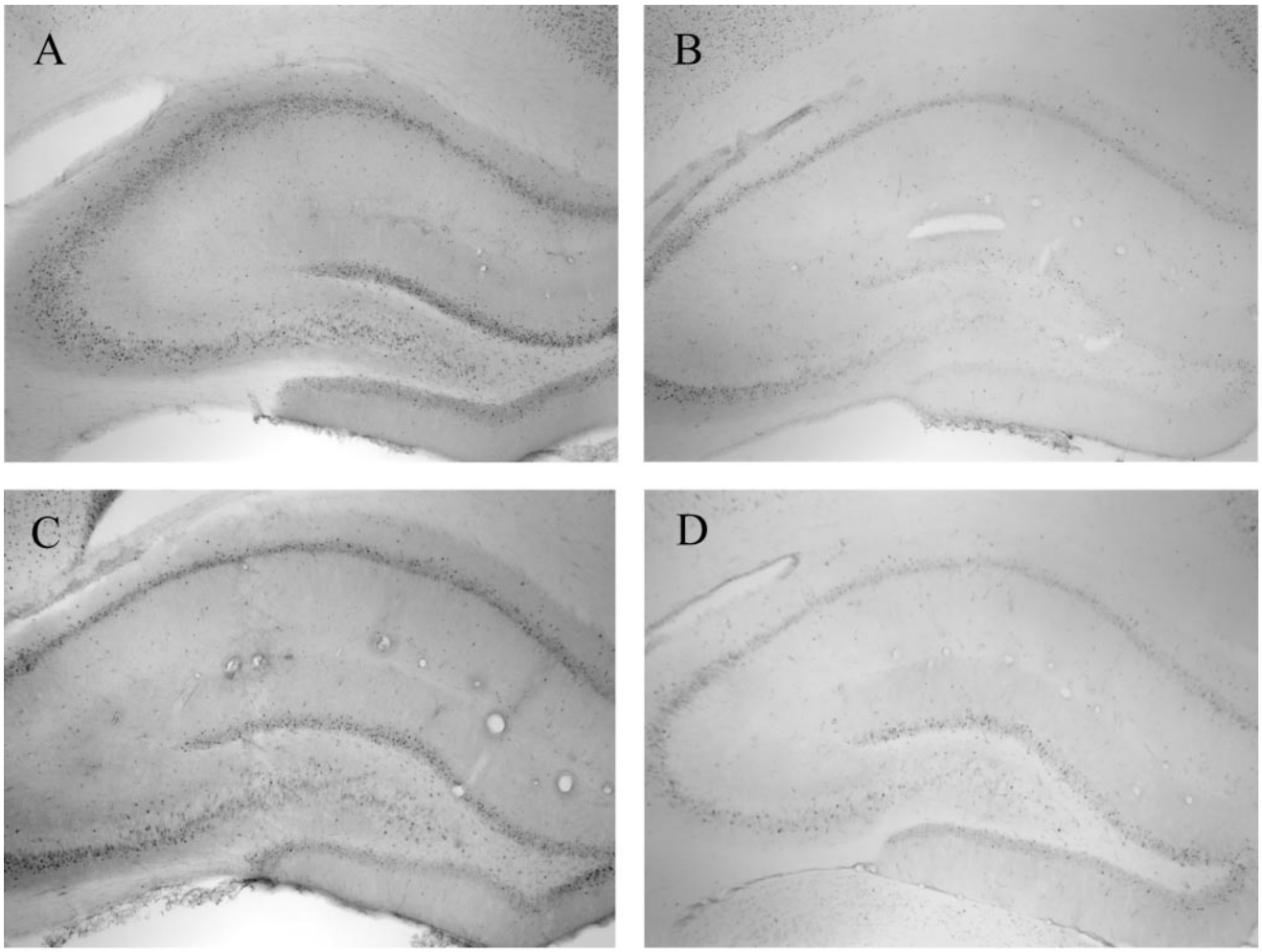

Figure 7. Representative c-Fos-IR immediately after training in the hippocampus of experimental (*A*) and motor control (*B*) rats. Representative c-Fos-IR 1 hr after training in the hippocampus of place learners (*C*) and response learners (*D*).

It has been proposed that the dorsomedial and dorsolateral striatum are involved primarily in stimulus–stimulus and stimulus–response associations, respectively (Devan and White, 1999; Devan et al., 1999; White and McDonald, 2002). Rats that adopted a response strategy had elevated pCREB in the dorsolateral striatum both immediately and 1 hr after learning compared with place learners, which is consistent with the hypothesis that the dorsolateral striatum has a prominent role in coding stimulus–response associations. In contrast, there was no difference in pCREB between place and response learners in the dorsomedial striatum immediately after training. By 1 hr after training, levels of pCREB in the dorsomedial striatum were significantly higher in response learners than in place learners. Thus, although the present findings indicate neuronal plasticity in the dorsolateral striatum during response learning, they do not support a prominent role for the dorsomedial striatum during place learning. Studies in which rats are trained to use a place strategy rather than to choose between at least two different strategies may result in a stronger test of the hypothesis that activity in the dorsomedial striatum is related to place learning.

It is well established that formation of long-term memory is dependent on protein synthesis (Davis and Squire, 1984), and current theories of the cellular mechanisms of memory formation indicate that expression of c-Fos and the transcription of late-effector genes may stabilize synapses and increase synaptic efficacy to subsequent stimuli (Morgan and Curran, 1991). Transient synapse formation, for example, is related to CREB phosphorylation after avoidance learning (O'Connell et al., 2000). Reports of learning-induced CREB phosphorylation indicate that the time courses of peak phosphorylation may vary. For single-trial aversively motivated learning, CREB phosphorylation may be biphasic, with peaks immediately and 3–6 hr after training, but there is disagreement as to whether the immediate increase is caused by the aversive foot shock alone or the learned association (Bernabeu et al., 1997; Stanciu et al., 2002). In contrast to the biphasic peaks, Taubenfeld et al. (2001) reported that inhibitory avoidance training caused CREB phosphorylation that began immediately and was sustained for up to 20 hr after training. Moreover, increased pCREB was reported 2 hr after inhibitory avoidance training (Cammarota et al., 2000) and 1–2 hr after exposure to a novel environment (Vianna et al., 2000). For incremental learning in the radial arm maze, CREB phosphorylation reportedly is elevated after the last training trial on days 4 and 8 (Mizuno et al., 2002), which indicates that activity is either sustained or occurs in relation to each bout of learning. Learning-related expression of c-Fos is generally reported to occur 1–2 hr after training (Curran and Morgan, 1995; Stanciu et al., 2002). A recent study shows that c-Fos is expressed in the hippocampus during radial arm maze training, and that inhibition of c-Fos by antisense oligonucleotide treatment impairs spatial memory for-

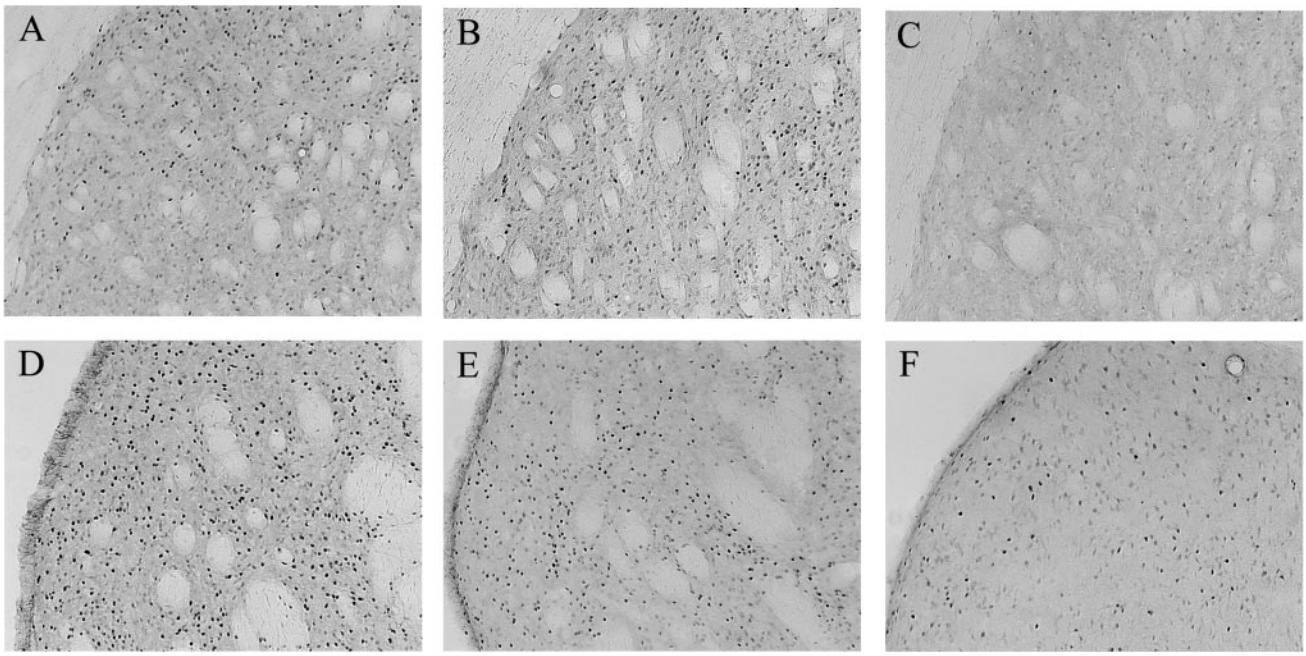

Figure 8. Representative c-Fos-IR in the dorsolateral striatum of experimental (*A*) and motor control (*B*) rats immediately after training and experimental rats (*C*) 1 hr after training. Representative c-Fos-IR in the dorsomedial striatum of experimental (*D*) and motor control (*E*) rats immediately after training and experimental rats (*F*) 1 hr after training.

mation (He et al., 2002). Sustained strategy- and region-specific activity of c-Fos and CREB 1 hr after training, therefore, is in the range of previous reports of the time courses of learning-related activity of transcription factors. The present results indicate that pCREB is increased throughout the dorsal hippocampus of place learners 1 hr after training, whereas c-Fos is expressed in CA1 and the dentate gyrus but not in CA3. There is evidence that CA3 may be active during pattern completion rather than spatial learning because of recurrent connectivity (Kazu et al., 2002). It remains to be determined, however, whether the differences between c-Fos expression and CREB phosphorylation after cross maze training are attributable to differences in their time courses of maximal activity or to other factors.

As evidence for multiple memory systems increases, theorists are beginning to form hypotheses about how these systems interact in the intact brain. Multiple memory systems may act independently, cooperatively, competitively, or in temporal sequence (Packard and Knowlton, 2002; White and McDonald, 2002). The present findings support the view that the hippocampus and striatum can act in parallel during acquisition of a task that can be solved by either hippocampus- or striatum-dependent strategies. Additional studies of incremental learning may be necessary to reveal conditions under which the hippocampus and striatum may act competitively or in temporal sequence. The present results suggest further that localized, functional measurements of transcription factors and other signaling proteins during memory formation are useful for describing relationships among multiple memory systems during normal cognitive processes in the intact brain.

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